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Dual Sec62/Ki67 immunocytochemistry of liquid-based cytological preparations represents a highly valid biomarker for non-invasive detection of head and neck squamous cell carcinomas

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Abstract

Background: Head and neck squamous cell carcinomas (HNSCC) are frequently diagnosed in advanced stages, which limits therapeutic options and results in persistently poor patient outcomes. The aim of this study was to use liquid-based swab cytology (LBC) in combination with dual immunocytochemical detection of migration and proliferation markers Sec62 and Ki67 in order to allow non-invasive early detection of HNSCC as well as to analyse the diagnostic validity of this method for predicting the malignancy of suspicious oral lesions.

Methods: 104 HNSCC patients and 28 control patients, including healthy patients ($n = 17$), papilloma ($n = 1$) and leukoplakia patients ($n = 10$), were included in this study. For all patients, an LBC swab followed by simultaneous immunocytochemical detection of Sec62 and Ki67 was performed. Immunocytochemical as well as cytopathological results were correlated with histological diagnoses and clinical findings.

Results: All HNSCC patients (100%) showed dual Sec62/Ki67 positivity, and all control patients except for the papilloma patient were negative for Sec62/Ki67 (96.4%), resulting in a 100% sensitivity and 96.4% specificity of Sec62/Ki67 dual stain for non-invasive detection of HNSCC. The positive predictive value was 99% and the negative predictive value was 100%. Sec62 expression levels showed a positive correlation with tumour de-differentiation ($p = 0.0489$).

Conclusion: Simultaneous immunocytochemical detection of Sec62/Ki67 using LBC represents a promising non-invasive and easy-to-apply tool for the early detection of HNSCC in routine clinical practice. This novel technique can help to avoid incisional biopsies and reduce the frequency with which general anaesthesia is used in diagnostic procedures in patients with suspicious oral lesions.

KEYWORDS
diagnostic biomarkers, head and neck squamous cell carcinoma, immunocytochemistry, Ki67, liquid-based cytology, Sec62

1 | INTRODUCTION

Squamous cell carcinomas of the oral, pharyngeal and laryngeal mucosa (head and neck squamous cell carcinomas, HNSCCs) represent up to 90% of all malignancies of the head and neck region. Chronic alcohol and nicotine consumption as well as high-risk human papillomavirus (HPV) infection, especially in oropharyngeal carcinomas, represent the major risk factors for the development of these tumours.[1] Since HNSCCs represent the 8th most common tumour disease worldwide, accounting for approximately 878,000 new cases and 444,000 deaths each year, those patients are frequently seen not only by otorhinolaryngologists but also by general practitioners, maxillofacial surgeons and dentists.[2] Over the past 15 years, the 5-year survival rate has only slightly improved to 66%; this is mainly due to the rapidly increasing proportion of HPV-associated HNSCCs[3] associated with a favourable outcome. Therapeutic options are still limited, with no major therapeutic breakthrough over the past 20 years, apart from the introduction of checkpoint inhibitors as a treatment option in recurrent and metastatic disease.[4] One major reason for this persistently poor prognosis is the significant diagnostic delay experienced by the majority of patients, who are predominately diagnosed at advanced stages. Almost half of the patients already show lymph node metastases at the time of diagnosis, so therapeutic options are significantly limited.[1,5] Even if the initial therapy is successful, almost half of the patients develop a tumour recurrence during the first 5 years of follow-up.[1,6,7] There is, therefore, an urgent need for screening approaches and/or methods for the early detection of HNSCC that include diagnostic biomarkers. So far, the findings presented in the literature for several of the potential biomarkers that have been proposed for early detection of HNSCC—including EGFR, Cyclin D1 and DNA methylation[8]—have been inconsistent; however, it should be noted that they were analysed in mostly small and heterogeneous patient cohorts. Hence, no diagnostic biomarker is currently established in clinical practice and neither recent NCCN (National Comprehensive Cancer Network)[9] nor European guidelines[10] recommend the use of biomarkers for diagnostic workup of suspicious oral lesions.

The SEC62 gene located at chromosomal region 3q26.2 encodes an endoplasmic reticulum (ER) transmembrane protein and is associated with the heterotrimeric SEC61 complex, which is responsible for the translocation of precursor proteins from the cytosol to the ER lumen and for regulating intracellular calcium homeostasis.[11,12] Under physiological conditions, Sec62 plays an essential role in the process of recovER-phagy in order to facilitate recovery from ER stress.[13] Additionally, Sec62 is involved in the transport of a subset of secretory and transmembrane protein precursors[14,15] and regulates cytosolic calcium levels through its interaction with Sec61.[16] SEC62 overexpression has been observed in various human cancers, including HNSCC,[17] cervical cancer,[18] breast cancer,[19,20] prostate cancer,[21] melanoma,[22] atypical fibroxanthoma,[23] hepatocellular cancer[24,25] and non-small cell lung cancer (NSCLC).[26] From a functional perspective, it was shown that Sec62 functions as a key regulator of tumour cell metastasis[17,19,20,26–28] and plays a role as a prognostic marker in NSCLC,[26] HNSCC,[17] melanoma,[22] ovarian cancer,[29] and breast cancer[20] with high expression levels being associated with poor outcomes. Additionally, high *SEC62* expression was associated with a higher risk of tumour recurrence in hepatocellular cancer.[25,30]

The nuclear protein Ki67 is a well-established proliferation marker that shows increased expression in almost all tumour entities. Our group has previously described the combinational use of Sec62 as a biomarker for tumour cell migration and Ki67 as a biomarker for tumour cell proliferation in previous studies of vulvar and cervical squamous cell carcinomas. It was shown that dual positivity of Sec62/Ki67 detected by immunocytochemistry in liquid-based cytological preparations (LBC) can be used as a sensitive predictor for malignant transformation of both dysplastic cervical and vulvar lesions.[18,31]

Against this background, this study aimed to use LBC for morphological analysis of suspicious lesions of the head and neck mucosa and combine this technique with dual immunocytochemical Sec62/Ki67 staining for early non-invasive detection of HNSCC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

In total, 132 patients were enrolled in this study. 104 patients had a histopathological diagnosis of HNSCC, located at the oropharynx ($n=54$), larynx ($n=21$), hypopharynx ($n=15$), and oral cavity ($n=14$). The tumour patient cohort comprised 80 male (82%) and 24 female patients (23%) with a mean age of 69 years. The control patients ($n=28$) showed a histopathological diagnosis of healthy mucosa without dysplasia ($n=17$), leukoplakia with low ($n=5$) to moderate dysplasia ($n=5$) and oral papilloma ($n=1$). Further clinical characteristics are shown in Table 1. For all included patients, biopsies and LBC samples were taken during a diagnostic panendoscopy under general anaesthesia. All patients were treated at the Department of Otorhinolaryngology at the Saarland University Medical Center (Homburg, Germany), with a median follow-up of 33 months. All patients gave their informed consent to participate in this study. All experiments were carried out in accordance with the Declaration of Helsinki and the relevant national and international ethical standards. This study was approved by the Saarland Medical Association ethics review committee (index number 218-10).

TABLE 1 Clinical characteristics of the included patients.

	Control patient	HNSCC	Total
Number of patients	28	104	132
Sex			
Male	22 (79%)	80 (77%)	102 (79%)
Female	6 (21%)	24 (23%)	30 (21%)
Median age (years)	65	69	69
Localisation			
Oral cavity	4 (14%)	14 (13%)	
Oropharynx	14 (50%)	54 (52%)	
Hypopharynx	4 (14%)	15 (14%)	
Larynx	6 (22%)	21 (21%)	
T-stage			
T1		29 (28%)	
T2		37 (36%)	
T3		21 (20%)	
T4		17 (16%)	
N-stage			
N0		50 (48%)	
N1		17 (16%)	
N2		28 (27%)	
N3		9 (9%)	
M-stage			
M0		98 (94%)	
M1		6 (6%)	
Grading			
G1		2 (2%)	
G2		47 (45%)	
G3		55 (53%)	
p16 + (IHC)		38 (37%)	
Therapy			
Surgery		22 (21%)	
Surgery + RT		25 (24%)	
Surgery + CRT		26 (25%)	
Primary CRT		25 (24%)	
Palliative CT		6 (6%)	

Abbreviations: CRT, chemoradiotherapy; CT, chemotherapy; HNSCC, head and neck squamous cell carcinomas; IHC, immunohistochemistry; RT, radiotherapy

2.2 | Liquid-based swab cytology

LBC samples were taken during diagnostic panendoscopy under general anaesthesia prior to surgical biopsy from the same area. Macroscopically suspect mucosal areas were gently wiped off using a Medscand Cytobrush (Cooper Surgical Inc). After that, the brushes were immersed and rinsed in a PreservCyt Solution vial (Hologic Inc.) and the cell suspension was transferred onto microscope glass slides using the ThinPrep®-system (Hologic Inc.) according to the manufacturer's instructions.

2.3 | Papanicolaou staining and morphological analysis of LBC preparations

For cytopathological analysis, Papanicolaou (Pap) stainings of LBC preparations were performed using a standard protocol. Since there are no standardised criteria for the cytological diagnosis of HNSCC, the established cytomorphological criteria for uterine cervical smears (Bethesda system)[32] were used due to comparable cytomorphological characteristics. Two technical assistants with wide experience in valuing Pap smears of the uterine cervix and one cytopathologist independently classified the specimens, according to the Bethesda system, into 4 categories: NILM (negative for intraepithelial lesion or malignancy), LSIL (low-grade squamous intraepithelial lesion), HSIL (high-grade squamous intraepithelial lesion), and SCC (squamous cell carcinoma).

2.4 | Immunocytochemistry (ICC)

After fixation of LBC microscope slides for 10 minutes in ethanol and drying for 20 minutes at room temperature, the slides were put in retrieval buffer (Tris/EDTA-buffer solution pH 9.0—Roche) for 20 minutes at 95°C for a heat-induced epitope retrieval. To block nonspecific protein binding sites, an incubation in 3% BSA (bovine serum albumin)-PBS (phosphate buffer solution; Sigma) for 30 minutes at room temperature was performed. Afterwards, slides were covered with the primary antibody cocktail containing Anti-Ki-67 (1:600 in 1% BSA/PBS; Ki-67 (mouse), DAKO M7240) and Anti-Sec62 (1:400 in 1% BSA/PBS; Sec62 (rabbit), abcam ab140644)) for 60 minutes at 37°C. After PBS (pH 7.2) washing, the slides were coated with the visualisation reagents alkaline phosphatase, horseradish peroxidase, DAB (3,3′-diaminobenzidine) substrate-chromogen solution, and fast-red chromogen solution using a CINtec PLUS kit according to the manufacturer's instructions (Roche). This process resulted in red and brown staining at the Sec62 and Ki67 antigen sites. This step was followed by washing with PBS (pH 7.2) and counterstaining with haematoxylin (Sigma). A two-step embedding procedure was performed using an aqueous embedding medium and Entellan (Sigma). Controls included a positive control for each staining series (provided with the CINtecPLUS kit) as well as a negative control by omission of the primary antibody.

2.5 | Immunohistochemistry (IHC)

For immunohistochemical analyses, formalin-fixed paraffin-embedded (FFPE) tissue samples of the tumours were obtained for all included patients. A Leica RM 2235 rotary microtome was used to obtain 3-µm sections which were transferred onto Superfrost Ultra

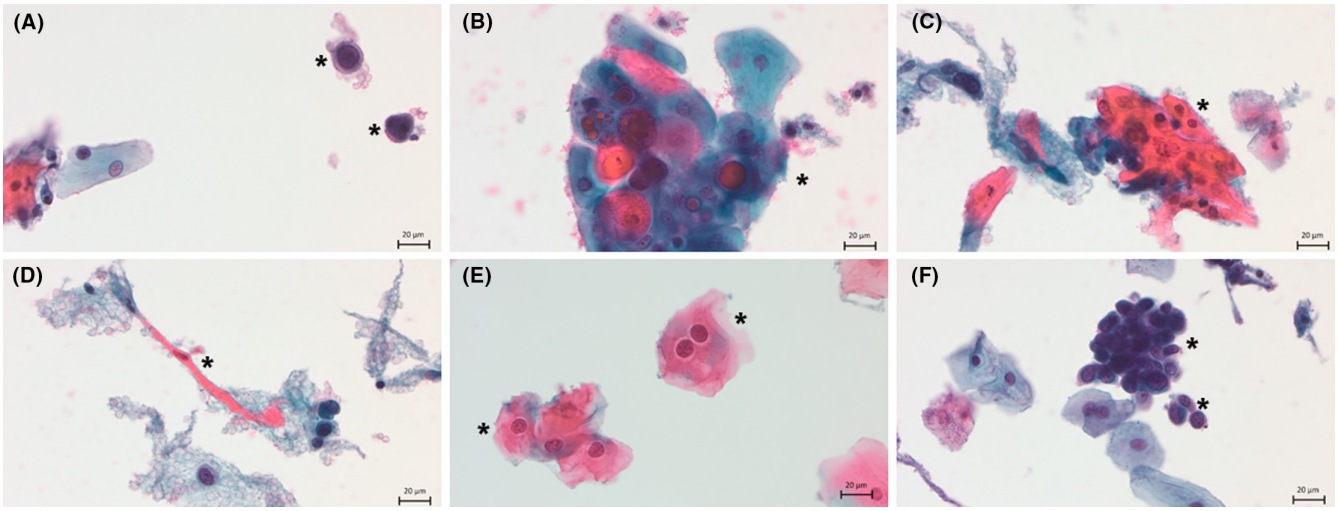

FIGURE 1 Cytomorphological characteristics of head and neck squamous cell carcinoma cells in liquid-based cytology preparations. (A) Increased nucleus-to-cytoplasmic ratio, (B) anisonucleosis, (C) hyperkeratosis, (D) tumour cell with elongated shape (ponytail pattern), (E) koilocytosis, (F) poorly differentiated tumour cells in a p16-positive case. In (A–F), lesional cells are marked with an asterisk (*). (A–F, Papanicolaou staining, 40× magnification)

Plus microscope slides (Menzel-Gläser) and dried in an incubator at 37°C overnight. After deparaffinisation, heat-induced epitope-unmasking was performed in 10 mM citrate buffer (pH 6.0). After cooling down to room temperature, unspecific protein binding sites were blocked by incubation in 200 mL PBS (Sigma) and 6 g Bovine serum albumin (BSA) under pH control (7.2) for 30 minutes at room temperature. Afterwards, simultaneous Sec62/Ki67 IHC staining was performed. First, the slides were incubated with the first primary antibody (1:600 in 1% BSA/PBS; Ki-67 (mouse), DAKO M7240, Santa Clara, CA, USA) overnight at 4°C. After heating to room temperature and visualisation using Streptavidin Alkaline Phosphatase and Chromogen Red with the Dako REAL™ Detection System Alkaline Phosphatase/RED (Dako) according to the manufacturer's instructions, unspecific protein binding sites were blocked again as described above. After that, the second primary antibody (1:1500 in 1% BSA/PBS; Sec62 (rabbit), abcam ab140644, Cambridge, UK) was applied and was incubated overnight at 4°C. The next day, a visualisation was performed as described above using the Dako REAL™ Detection System Alkaline Phosphatase/Chromogen brown (Dako). Finally, slides were counterstained with haematoxylin. Controls included a positive control for each staining series (uterine cervix squamous cell carcinoma) as well as a negative control by omission of the primary antibody. In addition, haematoxylin and eosin (HE) stains were prepared for each sample for morphological control using a standard protocol.

For p16 IHC, the previously deparaffinised and unmasked sections, as described above, were incubated with the primary antibody (1:4000 in 1% BSA/PBS; p16 (mouse) Abcam 201,980) for 60 minutes. After washing with PBS, the secondary antibody was added for 30 minutes. After another washing step with PBS, visualisation was performed via streptavidin alkaline phosphatase and chromogen red as well as counterstained with haematoxylin as described above. All IHC specimens were evaluated independently by five examiners (one pathologist, two otorhinolaryngologists, and two technical assistants with wide experience in evaluating immunostainings of the head and neck region).

2.6 | Evaluation of immunocytochemistry and immunohistochemistry Sec62/Ki67 dual stain

For the evaluation of ICC and IHC Sec62/Ki67 dual staining, the immunoreactivity of squamous cells was only considered and valued as positive if at least one cell per sample showed a cytoplasmic red (Sec62) and nuclear brown (Ki67) staining signal. Cases without dual-positive squamous cells were considered negative. In Sec62/Ki67 positivity cases, Sec62-immunoreactivity was additionally quantified using a modified immunoreactive score (IRS) according to Remmele and Stegner.[33] Here, IRS values ranging from 0 to 4 were calculated based on the staining intensity of squamous cells (0: no reaction, 1: mild reaction, 2: moderate reaction, 3: intense reaction). Again, all Sec62/Ki67 dual staining IHC and ICC preparations were evaluated independently by five examiners as described for p16 IHC.

2.7 | Statistical analysis

For statistical analyses, Prism V9 software (GraphPad, Boston, MA, USA) was used. The D'Agostino & Pearson omnibus normality test, Anderson-Darling test, Shapiro–Wilk test, and Kolmogorov–Smirnov test were used to determine if datasets follow a Gaussian distribution in each comparison. Gaussian distribution was only assigned if the data sample passed two or more of the aforementioned normality tests. If the data showed a normal distribution, parametric tests were performed (two-tailed unpaired *t*-tests). If the data showed no normal distribution, non-parametric tests were

applied (Mann-Whitney-*U* test). For survival analysis, a log-rank test was used. p values <0.05 were considered statistically significant ($\alpha=0.05$).

3 | RESULTS

3.1 | Morphological characteristics and Bethesda classification of head and neck liquid-based cytology preparations

First, PAP-stained LBC preparations from all included patients were morphologically evaluated. A variety of cytomorphological features characteristic of squamous cell carcinomas were found in samples from histologically proven HNSCC patients (see Figure 1), including increased nucleus-to-cytoplasmic ratio, anisonucleosis, hyperkeratosis, ponytail-like tumour cells, and koilocytosis. LBC preparations from all included patients predominately showed single cell layers and were eligible for further analysis.

LBC preparations were then classified according to the Bethesda system. As shown in Table 2, 97% (101/103) of patients with a histological diagnosis of SCC were also classified as SCC based on cytomorphology. Patients with histologically healthy mucosa were cytologically predominately classified as NILM (94%, 16/17) while leukoplakia patients with various grades of dysplasia were classified as LSIL (80%, 8/10) and HSIL (20%, 2/10). One papilloma patient that was included in our study was cytologically valued as LSIL.

3.2 | Diagnostic accuracy of Sec62/Ki67 dual positivity for detecting HNSCC

In the immunocytochemical analysis of LBC preparations, all of the included 104 HNSCC patients (100%) showed a Sec62/Ki67 positivity in cells that were morphologically identified as cancer cells. In contrast, 27 of 28 control patients showed no Sec62/Ki67 dual-positive cells in the LBC preparations (96.4%). For a single patient in the control group who was histologically diagnosed with a tonsil papilloma, dual-positive lesional cells were found in the respective ICC slide; however, they showed no cytomorphological signs of malignancy. Thus, the sensitivity of immunocytochemical Sec62/i67 dual positivity for the detection of squamous cell carcinoma in our cohort of $n=132$ patients was 100%, with a specificity of 96.4%, a positive predictive value of 99% and a negative predictive value of 100%.

When comparing Sec62/Ki67 ICC with IHC staining of respective FFPE tissue samples, concordant results were found for all cases. Exemplary ICC, IHC and H&E images of one p16-positive and one p16-negative SCC case are shown in Figure 2.

3.3 | Correlation of *SEC62* expression with histopathological characteristics and clinical data

In the next step, we correlated the intensity of immunocytochemical *SEC62* expression in the SCC cases with the grade of tumour cell differentiation (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated) and found a gradual increase in *SEC62* expression along with tumour cell de-differentiation. Significantly higher levels of *SEC62* were detected in G3 compared to G1 cases ($p=0.0489$). Additionally, there was a trend towards higher levels in G2 compared to G1 cases ($p=0.0824$; Figure 3A).

When correlating immunocytochemical *SEC62* expression in lesional cells of the SCC cases with the presence of lymphatic metastasis (N0, no lymph node metastases; N+, one lymph node metastasis), no significant difference between N0 or N+ was found ($p=0.1436$, Figure 3B). Additionally, a potential influence of smoking status (non-active smokers vs active smokers) on *SEC62* expression was analysed but showed no significant difference (Figure 3C; $p=0.5277$). Also, p16 expression did not show a significant correlation with *SEC62* expression levels (data not shown).

3.4 | Prognostic relevance of *SEC62* expression in head and neck cancer liquid-based cytology preparations

Furthermore, as Sec62 is known to be a relevant indicator of poor outcomes in a variety of human cancers, we analysed a potential correlation of immunocytochemical *SEC62* expression in lesional cells with

TABLE 2 Correlation of histomorphological and cytomorphological diagnoses.

	Bethesda classification of LBC preparations				
Histological diagnosis	**NILM**	**LSIL**	**HSIL**	**SCC**	**∑**
SCC			3	101	104
Healthy mucosa	16	1			17
Leukoplakia + low-grade dysplasia		5			5
Leukoplakia + moderate dysplasia		3	2		5
Papilloma		1			1

Abbreviations: HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; SCC, squamous cell carcinoma

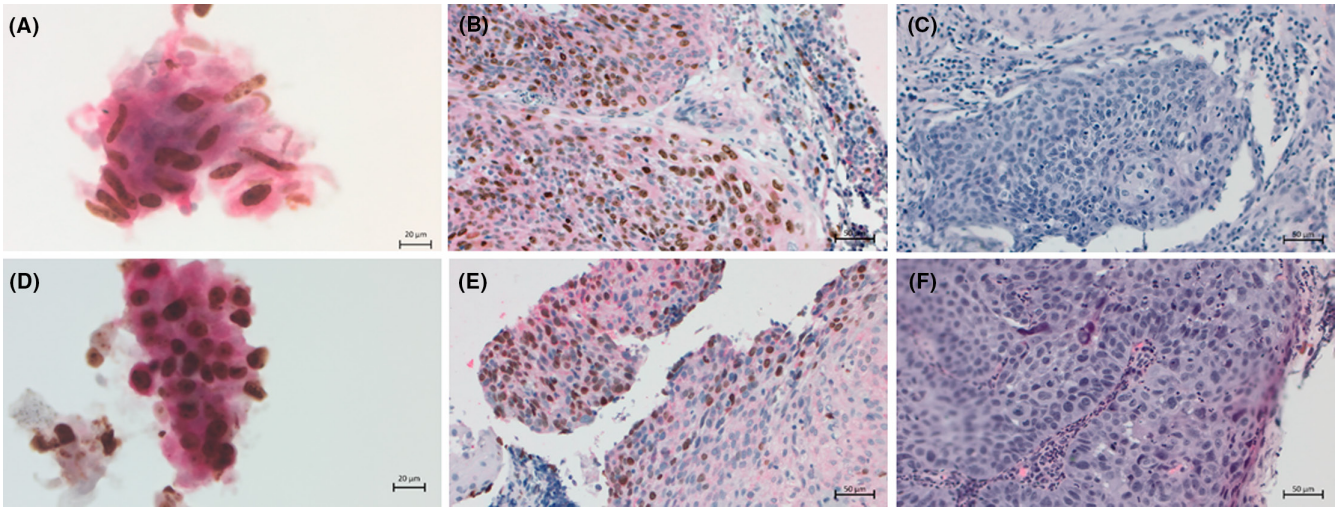

FIGURE 2 Immunocytochemical Sec62/Ki67 dual staining of head and neck squamous cell carcinoma liquid-based cytology preparations in one p16-positive (A) and one p16-negative (D) patient and corresponding immunohistochemically (B, E) and haematoxylin and eosin (C, F) stained formalin-fixed paraffin-embedded tissue preparations. In (A, B, D, E), Sec62 is marked by a red cytoplasmic signal and Ki67 is marked by a brown nuclear signal. (A, D, 40× magnification; B, C, E, F, 20× magnification)

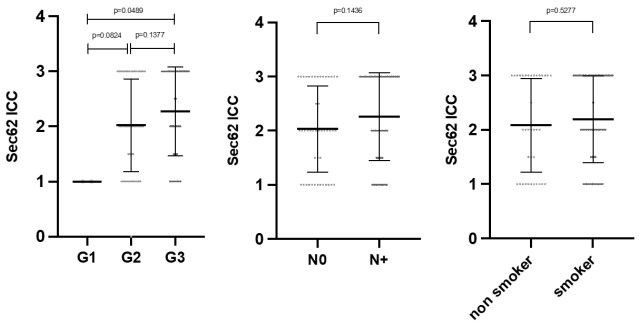

FIGURE 3 Correlation of *SEC62* expression detected by immunocytochemistry with tumour differentiation, lymph node status and smoking behaviour. (A) Correlation of *SEC62* expression with tumour differentiation (histological tumour grading), (B) lymph node status (N0, no lymph node metastasis; N+, one or more lymph node metastases), and smoking behaviour (C). In (A–C), the median is indicated by a horizontal line, and the standard deviation is illustrated by error bars. Every dot represents one patient. (A–C, Mann-Whitney U test, *p*-values are indicated in the figures)

overall survival (OS) and disease-specific survival (DSS) for all SCC patients. *SEC62* expression levels showed no statistically significant influence on OS or DSS when comparing a Sec62 high with a Sec62 low group, separated by the median of Sec62 IRS (see Figure 4A,B). In contrast, patients with a p16-positive tumour tested by IHC showed a superior overall survival compared to p16-negative patients ($p=0.0092$, log-rank test, Figure 4C).

4 | DISCUSSION

HNSCCs represent the 8th most common cancer worldwide, resulting in approximately 444,000 deaths each year.[2] Despite the fact that a rapidly increasing proportion of HPV-positive HNSCCs are associated with a better prognosis, the 5-year survival rate remains persistently low (currently at 66%).[3] Due to the fact that the majority of patients are diagnosed at advanced stages and an earlier diagnosis could significantly improve patients' outcomes and treatment options, there is an urgent need for new diagnostic methods, including diagnostic biomarkers for the early and valid detection of HNSCCs.

In our study, we analysed the diagnostic accuracy of immunocytochemical Sec62/Ki67 dual staining of liquid-based cytological swab preparations for detecting HNSCCs and discriminating them from non-neoplastic as well as precancerous non-invasive lesions of the head and neck mucosa. We found a diagnostic sensitivity of 100%, specificity of 96.4%, positive predictive value of 99% and negative predictive value of 100% for diagnosing invasive carcinoma in a cohort of $n=132$ patients, underlining the high potential of this technique for non-invasive cytology-based detection of SCC of the head and neck.

Currently, surgical probe excisions of oral lesions suspicious for HNSCC represent a well-established clinical gold standard in diagnostic work-up, which requires general anaesthesia in most cases. However, it is well known that this invasive procedure has its limitations and does not reliably exclude false-negative results. For example, Pentenero et al. have shown that up to 24% of oral squamous cell carcinomas are not diagnosed using scalpel biopsy.[34] In contrast, brush biopsies, as performed in our study, are a convenient, non-invasive, cost-effective, and relatively painless alternative method of collecting lesional cells from the oral and pharyngeal mucosa that can be used for a variety of different diagnostic purposes, including cytomorphological analysis, immunostainings, in-situ hybridisation,[35] PCR[36] and HPV testing.[37] Several studies found a high diagnostic accuracy of LBC for detecting HNSCC, with a sensitivity ranging from 95.6% to 100% and a specificity ranging from 68.8% to 86.5%.[38–40] Nonetheless, diagnostic

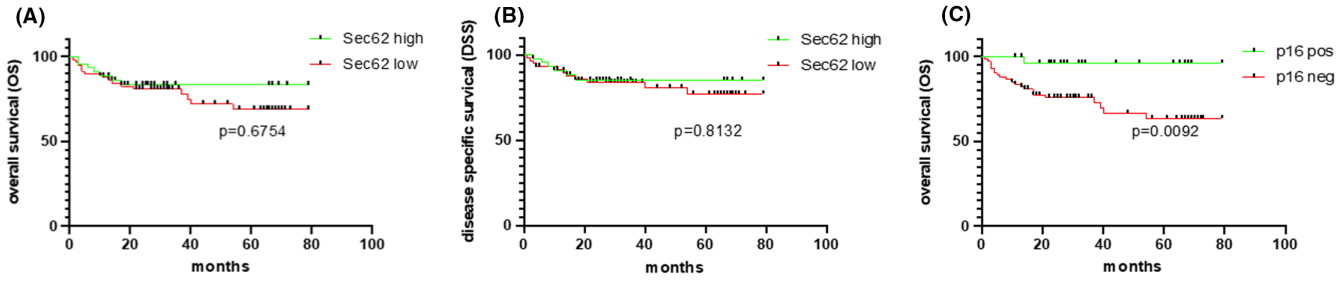

FIGURE 4 Prognostic relevance of immunocytochemically detected *SEC62* expression and p16 status (immunohistochemistry). Kaplan-Meier analysis of patient overall survival (A) and disease specific survival (B) depending on *SEC62* expression (red, low *SEC62* expression, IRS 0-6; green, high *SEC62* expression, IRS 7-12). (C) Kaplan-Meier analysis of patient overall survival depending on p16 status (green, positive; red, negative). (A–C, log-rank test, censored data are indicated by black dots)

accuracy needs to be further increased in order to allow a transfer to clinical practice, especially if the cytological diagnosis may result in a therapeutic intervention. One potential way to enhance the diagnostic performance of LBC in HNSCC diagnostics is the immunocytochemical detection of protein biomarkers (e.g. Sec62 and Ki67, as used in our study).

In contrast to Ki67, which has, for decades, been used as an established proliferation biomarker in immunocytological as well as immunohistological diagnostics[41], the role of Sec62 as a potential biomarker in human cancer only began to be investigated in the last 10 years.[27] The functional role of Sec62, which acts as a migration- and invasion-stimulating oncogene, raises the stress tolerance of cancer cells and is associated with poor patient outcomes when markedly overexpressed, is becoming increasingly understood; however, there have only been a few studies evaluating the potential use of Sec62 as a diagnostic biomarker for cytological cancer diagnostics. In a pilot study comprising LBC specimens from 45 women with vulva pre-cancerous and cancerous lesions, Takacs et al. demonstrated that immunocytochemical Sec62/Ki67 dual-stainings achieve a sensitivity of 100% and a specificity of 76.9% for detecting squamous cell carcinomas of the vulva.[31] In a comparable study, the same group applied dual Sec62/Ki67 ICC to LBC samples of $n = 100$ women with histological diagnoses ranging from non-dysplastic cervical mucosa, CIN1, CIN2, and CIN3 to cervical SCC.[18] Here, Sec62/Ki67 dual-positive cells were only found in CIN2, CIN3, and SCC patients, with the highest percentage of positive patients in the CIN3 and SCC groups (100%, each). For detecting CIN3+ cervical lesions, dual Sec62/Ki67 ICC achieved a sensitivity of 100% and a specificity of 92%. The results of both of the aforementioned studies are consistent with our findings, the only difference being that dual-positive cells were partially detected in LBC samples from highly dysplastic vulva and cervical lesions (HSIL resp. VIN3/CIN3). As in our head and neck study cohort, no case with histological diagnosis of high-grade intraepithelial neoplasia was included, and we can only speculate on Sec62/Ki67 immunoreactivity in those cases. However, premalignant lesions in the oral, pharyngeal and laryngeal region are very rare, with less than 5% of SCC arising from clinically apparent precursor lesions,[42] so that the clinical relevance of this patient group is much lower compared to the vulva and cervical region.

Considering the correlation of *SEC62* expression detected by ICC with clinical and histopathological characteristics, we found a significant positive correlation of Sec62 protein levels with tumour cell de-differentiation as well as a tendency towards higher levels in metastasised tumour patients. Consistently, comparable results for an association of *SEC62* expression with tumour differentiation were reported for NSCLC.[26] In addition, a positive correlation of higher Sec62 levels with the occurrence of lymph node metastases was found in other cancer entities, including melanoma,[22] HNSCC,[17] and NSCLC.[26] These results, together with our data, strengthen the clinical relevance of the *SEC62* oncogene for tumour aggressiveness and metastatic spread. However, we found no significant influence of *SEC62* expression on the overall survival and disease-specific survival of the included HNSCC patients, which is contrary to previous reports of a worse outcome of melanoma,[22] HNSCC,[43] NSCLC,[26] and breast cancer patients[20] along with higher *SEC62* expression levels. Potential reasons for this discrepancy may include the limited number of patients and relatively short median follow-up in our study (33 months), as well as the use of the ICC technique for Sec62 detection—the previous studies described here used IHC to analyse *SEC62* expression levels. However, we also found no significant influence of *SEC62* expression on survival when we used Sec62/Ki67 IHC staining to quantify *SEC62* expression; thus, patient-related factors seem to be the main reason for this observation.

From a critical point of view, there are some limitations of our study that need to be discussed with regard to data interpretation and transferability to clinical practice. First, though liquid-based swab cytology represents a highly valid, cost-effective, and time-effective tool for the diagnostic work-up of suspicious oral lesions, it is not widely used in the everyday clinical practice of otorhinolaryngologists, maxillofacial surgeons or dentists, such that a minority of practitioners are even aware of this method.[44] Furthermore, cytomorphology of the head and neck region can be challenging even for experienced pathologists, which limits the transferability of this technique to clinical practice.[45] While in our pilot study swabs were taken during diagnostic panendoscopy under general anaesthesia to ensure that the same region that was biopsied was also swiped off with the cytobrush, this technique is

primarily intended to be used in an outpatient setting during the examination of a patient who is awake. Hence, we cannot completely exclude the possibility that patient-related factors such as low pain and gagging tolerances can potentially limit cytological sampling, though we rarely see this in everyday practice, as cytological swab sampling is comfortable for most patients. Nonetheless, we also included patients with hypopharyngeal and laryngeal lesions that are presumably not suitable candidates for swab cytology diagnostics during a routine clinical examination. Hence, the technique, as proposed in this study, is limited to lesions that can be conveniently reached with a cytobrush when the patient is awake.

When evaluating the ICC LBC specimens, some tumour cells showed a tendency to adhere to each other, and they often presented in clusters, which can lead to difficulties in interpretation. Due to the fact that Sec62 is located in the cytoplasm, atypical cancer cells lacking cytoplasm (so-called naked or bare nuclei) can hardly be identified as dual-positive, even if they are morphologically highly suspicious for malignancy. Overall, these morphological characteristics were observed only in single cases and were limited to single cells rather than to the entire slide. Finally, the otorhinolaryngologist resp. head and neck surgeon and the cytopathologist must discuss whether, for an individual case, LBC swab cytology is sufficient for diagnostic work-up or if an additional incisional biopsy will be necessary, especially once therapeutic consequences are considered. A recent report on so-called "cytology-based cancer surgery" of the head and neck showed that LBC of the head and neck region is not only a subordinated, additional diagnostic tool but can also be of use in head and neck cancer surgery and in the therapeutic management of HNSCC patients.[46]

5 | CONCLUSION

This is the first clinical study investigating Sec62/Ki67 dual staining on liquid-based cytological specimens from suspicious lesions of the head and neck mucosa. Taken together, our results indicate that dual immunocytochemical detection of Sec62/Ki67 in LBC swab preparations can be used for non-invasive detection of head and neck squamous cell carcinoma with a high degree of diagnostic accuracy. Additionally, *SEC62* expression levels in lesional cells detected by ICC can provide initial evidence for the clinical behaviour of the tumour.

AUTHOR CONTRIBUTIONS

JPK and ML wrote the paper, conceived and designed the analysis, collected the data, contributed data and analysis tools and performed the laboratory work and the analysis with SS and BL. SK and HR helped with the laboratory work. MW performed the histological examination. BS and EFS contributed to the study design and concept. LM critically examined the manuscript and evaluated and corrected it. All authors participated in writing and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest declared.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available on request from the corresponding author. The data are not publicly accessible due to privacy or ethical restrictions.

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